# PREPARATION OF AVIAN RIBOSOMES WITH LOW LEVELS OF CONTAMINATING ELONGATION FACTORS

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## 1. Introduction

The preparation of eukaryotic ribosomes with minimal contaminating activity for assaying of elongation factors usually requires rather extensive procedures [1,2].

Recently it was reported that rat liver rough microsomes can be dissociated in a medium of high ionic strength into ribosomal and membrane components by reaction of nascent polypeptides with puromycin. The released ribosomal components can be isolated as subunits [3,4].

Earlier it was observed that when *E. coli* ribosomes were separated into subunits, elongation factor EF-G was quantitatively removed [5]. It seemed possible that these facts taken together provided the basis of a simplified procedure for the isolation of chicken liver ribosomal subunits exhibiting low levels of elongation factor activity. This was found to be the case, and the results are presented in this paper.

## 2. Materials and methods

Chickens (age 50–60 days) were killed by decapitation and the livers excised immediately and placed in ice. The chilled material was disrupted in a Braun meat grinder at a setting of 2. This and all subsequent operations were carried out in the cold (0–4°C).

The minced tissue was suspended (2 ml/g tissue) in Buffer I (0.050 M Tris–HCl, pH 7.4, 0.025 M HCl, 0.005 M $MgCl_2$, 0.002 M dithiothreitol (DTT) and 0.34 M sucrose) and homogenized in a Manton-Gaulin Laboratory Homogenizer at 500 psi.

The initial homogenate was centrifuged at 1000 *g* for 30 min. The supernatant solution was centrifuged successively at 13 000 *g* for 45 min. The resulting supernatant solution was centrifuged at 100 000 *g* for 4 hr to yield a microsomal pellet. The microsomes were then suspended in Buffer II (0.050 M Tris–HCl, pH 7.4, 0.025 M KCl, 0.005 M $MgCl_2$, 0.002 M DTT and 0.025 M sucrose).

Ribosomal subunits were prepared from the microsomal fraction by direct reaction with puromycin in high salt as described by Adelman et al. [3]. The reaction mixtures contained one fifth volume of microsomal suspension and 0.050 M Tris–HCl, pH 7.4, 0.500 M KCl, 0.004 M $MgCl_2$, 0.002 M DTT and 0.001 M puromycin. The mixture was incubated at 37°C for 15 min and at 25°C for an additional 15 min. One ml aliquots of the reaction mixture were than applied to 5–20% (w/w) linear sucrose gradients layered over a 40% sucrose cushion and centrifuged at 27 000 rpm for 3.5 hr at 20°C (Beckman SW 27). Fractions were collected using an Isco Model D Gradient Fractionator, and the UV-absorbing material was analyzed using a Beckman Acta III spectrophotometer with Helma Flow cells (path length 2 mm). Regions of the gradients containing the subunits were pooled separately and were then centrifuged at 100 000 *g* for 16 hr to ensure complete pelleting of both components. The ribosomal subunits were suspended in Buffer II.

For comparison of factor activity, salt washed ribosomes were prepared. The microsome suspension was made 1.3% with respect to sodium deoxycholate and layered on discontinuous gradients (5 ml of 0.7 M sucrose, 5 ml of 2.0 M sucrose) [6,7]. The ribosomes were collected by centrifugation for 17 hr at 150 000 *g* and suspended in high salt buffer (0.050 M Tris–HCl, pH 7.4, 0.500 M KCl, 0.005 M $MgCl_2$, 0.002 M DTT and 0.25 M sucrose). The suspension was clarified by centrifugation at 10 000 *g* for 10 min and the ribosomes were collected by centrifugation at 198 000 *g* hr. Final suspension of washed ribosomes was in Buffer II. Prior to assay, both subunit and washed ribosome preparations were clarified by centrifugation at 10 000 *g* for 30 min.

Total ribosomal release was analyzed by centrifugation through discontinuous sucrose gradients (as described) after incubation in the presence of varying amounts of KCl and puromycin. Quantitation of ribosomal material was based on the determination of RNA content of each pellet by the method of Fleck and Munro [8].

GTPase activity throughout the continuous sucrose gradients was measured as described by Gordon [9] under the ionic conditions of the gradients.

## 3. Results

The effects of KCl and puromycin on the release of ribosomes from the microsomal preparation are shown in table 1. Greater than 80% of total ribosomal material was released in the presence of 0.5 M KCl when compared to sodium deoxycholate-treated controls. This high salt release was little effected by either incubation at 37°C or by inclusion of puromycin.

Table 1
Analysis of ribosomal release after treatment of microsomes with KCl and puromycin

| KCl | − Puromycin | | + Puromycin | |
|---|---|---|---|---|
| | 0°C | 37°C | 0°C | 37°C |
| 0.025 M | 21 | 18 | 18 | 20 |
| 0.100 M | 52 | 64 | 59 | 75 |
| 0.500 M | 81 | 84 | 84 | 86 |

Data expressed as percentage of detergent releasable RNA. Samples were incubated for 15 min at temperature indicated.

Increasing the KCl concentration above 0.5 M had no further effect on release (data not shown).

To examine the extent of ribosomal subunit release, we analyzed on continuous sucrose gradients microsomal material incubated under varying conditions of KCl and puromycin concentrations. Those results obtained by incubation at 37°C with low KCl–puromycin, high KCl alone and high KCl–puromycin are shown in fig. 1. Although the data from table 1 indicate no effect of puromycin on total release of ribosomal material, it is apparent (fig. 1) that the addition of puromycin was necessary for the maximal dissociation into subunits. Integration of the areas under the 60 S and 40 S O.D. profiles showed a 1.8-fold increase in

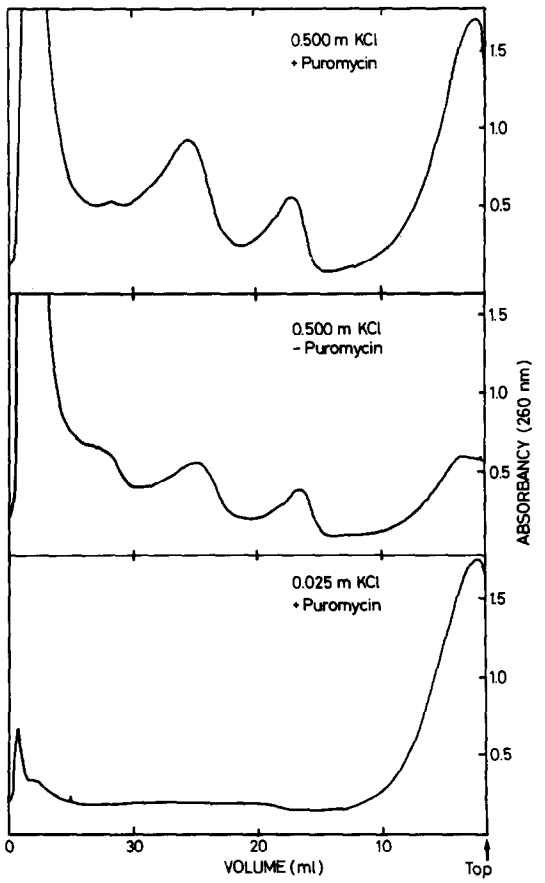


Fig. 1. Effect of KCl and puromycin on release of ribosomal microsomes. Each sucrose gradient was loaded with equal amounts of microsomal material (0.74 mg RNA). Sedimentation is from right to left.

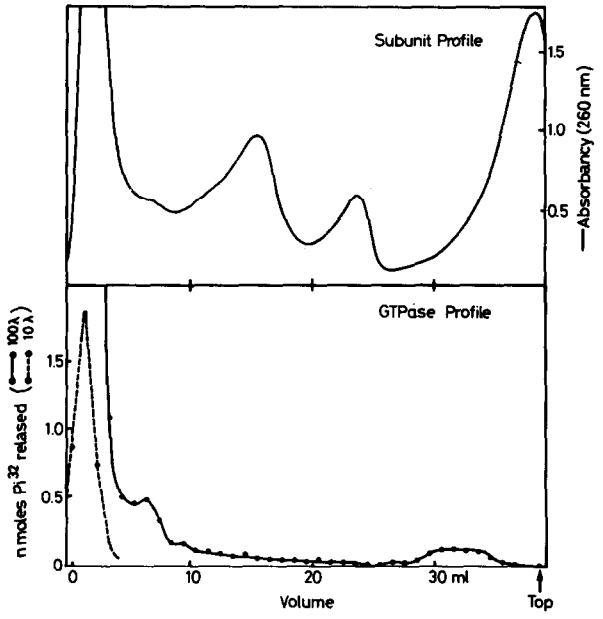


Fig. 2. Measurement of membrane contamination of ribosomal subunits isolated on sucrose gradients.

Table 2
Elongation factor activity of ribosome preparations

| Reaction mixture | Puromycin-released subunits 100 μg | 0.5 M KCl washed ribosomes 100 μg |
|---|---|---|
| | cpm | cpm |
| Complete | 16 750 | 16 500 |
| EF-1 omitted | 186 | 480 |
| EF-2 omitted | 53 | 380 |
| EF-1 + EF-2 omitted | 45 | 150 |

Poly(U)-directed polyphenylalanine synthesis was carried out in 0.250 ml reaction mixtures containing 0.05 M Tris–HCl, pH 7.4, 0.08 M $NH_4Cl$, 0.01 M $MgCl_2$, 0.01 M DTT, 250 μg poly(U), 0.001 M GTP, 100 μg [$^{14}$C]Phe–tRNA (35.5 pmoles of [$^{14}$C]Phe(specific activity 513 mCi/mM)) and saturating amounts of rat liver elongation factors EF-1 (5 μg) and EF-2 (10 μg). Elongation factors were prepared according to Felicetti and Lipmann [10] and were a generous gift from Dr. L. Felicetti. Polyphenylalanine synthesis was determined as hot TCA-insoluble radioactivity [10] and measured in an ambient temperature Nuclear Chicago Biocap III. The efficiency for $^{14}$C was 90%.

the amount of subunits obtained in the presence of puromycin over that released by 0.5 M KCl alone.

The large peak of absorbance at the bottom of the gradients is due to light scattering by released membrane material trapped above the 40% sucrose cushion. At low salt, where there is little release of ribosomes, the heavier membrane material pelleted through the cushion [4]. As a measure of 'cleanliness' of the ribosomal subunits, GTPase activity was measured throughout the gradient. As shown in fig. 2, the GTPase activity was associated with the membrane fraction and little activity was detectable in the regions of the 40 S and 60 S subunits. This was taken to indicate little contamination of the subunits by membrane material.

We then determined whether ribosomal subunits prepared using the high salt-puromycin procedure would indeed exhibit low levels of elongation factor activity. For comparison, we analyzed elongation factor activity of detergent-released, salt-washed ribosomes.

As shown in table 2, both preparations had comparable activity in reaction mixtures containing equal amounts of ribosomes and saturating elongation factors. Omission of EF-1 or EF-2, either separately or together, shows that the remaining elongation factor levels are reduced to minimal proportions in the subunit preparation and are less than the corresponding levels in the high salt-washed ribosomes.

Finally, it was found that the addition of degraded soluble RNA (600 μg/ml) [11] both during preparation of microsomes and during puromycin incubation results in subunits which have intact RNA species (data not shown) although no detectable differences in activity were observed.

## 4. Discussion

The results presented here show that chicken liver ribosomes are released and dissociated into subunits by incubation in the presence of high salt and puromycin.

The isolated ribosomal subunits are active in polyphenylalanine synthesis and exhibit minimal elongation factor activity.

Under our conditions of microsome isolation the procedure yields 1.5 mg of 60 S subunits and 0.6 mg

of 40 S subunits per gram (wet weight) of liver.

In conclusion, the procedure does indeed provide a direct and simplified method for obtaining clean ribosomes for analysis of elongation factor activity.

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